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Environmental control of the mucosal immune system

Goverse, G.

2015

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citation for published version (APA)

Goverse, G. (2015). *Environmental control of the mucosal immune system*. [PhD-Thesis - Research and graduation internal, Vrije Universiteit Amsterdam].

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English Summary

The mucosal immune system has the responsible task to maintain mucosal homeostasis, allowing tolerance against harmless antigens, derived from diet or commensal bacteria, but effective against pathogens and pathogenic antigens. Several environmental factors, such as dietary metabolites and microbial derived products, which are present in the intestinal lumen and thus in close proximity with the mucosal immune system, have been indicated as factors that can influence this homeostasis. Moreover, the host is able to regulate the composition of the microbiota present in the intestines with the production of e.g. defensins and mucus¹⁻³, while the microbiota influences the available nutrition for the host by diet fermentation^{4,5}. On top of all this, the nutritional intake of an individual also affects the composition of the microbiota⁶⁻⁸. Therefore, the regulation of the mucosal immune system is complex and can either be directly or indirectly modulated by various environmental factors.

The effect of vitamin A on the mucosal immune system.

A crucial role for vitamin A in the regulation of various processes that mediate the mucosal homeostasis are well documented. For instance, the tolerogenic phenotype of dendritic cells (DCs) within the mucosal immune system is dependent on the formation of the active metabolite of vitamin A, retinoic acid (RA), produced by intestinal epithelial cells⁹⁻¹¹. Consequently, these DCs can in turn produce high amounts of RA, which induces regulatory T-cells, IgA producing B-cells and gut homing molecules, allowing the lymphocytes to migrate to the lamina propria of the small intestines¹²⁻¹⁴. Together, these RA dependent processes control the tolerogenic functions of the mucosal immune system.

In this thesis we demonstrated additional aspects of the mucosal immune system that are regulated by dietary vitamin A. We show in chapter 2 that besides DCs also the differentiation of intestinal macrophages is affected by vitamin A. Although it has been reported that the production of pro-inflammatory cytokines in macrophages and DCs can be blocked by RA¹⁵⁻¹⁷, we established that the expression of Dectin-1 on intestinal macrophages was also dependent on RA. With the expression of the Dectin-1 receptor macrophages exhibited the plasticity to rapidly switch their phenotype upon ligation, which is needed during epithelial disruption and inflammation. Although contradictory results were obtained in Dectin-1 deficient mice, which received DSS induced colitis^{18,19}, links have been made with genetic alterations in the Dectin-1 gene in inflammatory bowel disease (IBD) patients^{19,20}. Dectin-1 is a C-type lectin receptor that upon binding will induce the production of pro-inflammatory cytokines. Whereas Dectin-1 has been shown to recognize bacterial antigens, its receptor is mainly known for its binding to fungi^{21,22}. Because the sequencing techniques, which are used for the analysis of the microflora composition present in the feces of the intestines, are not properly designed for fungi analysis,

studies so far may have missed the role of fungi. In addition, fungi are much bigger in mass compared to bacteria, therefore, although the numbers of fungi are much smaller, they might still have a significant contribution to the homeostasis of the host ²³. In conclusion, this indicates that regulation of the mucosal immune system via fungal signaling should not be underestimated.

Furthermore, a critical role for vitamin A in the formation of secondary lymphoid organs during embryogenesis was shown by our group. Lymphoid organogenesis depends on the clustering of lymphoid tissue inducer (LTi) cells, which belong to the group 3 innate lymphoid cells (ILCs). RA induced the expression of CXCL13 by stromal organizer cells, which recruited LTi cells for the initiation of cluster formation ²⁴. Subsequently the levels of maternal vitamin A further influenced the maturation of ILC progenitors into mature LTi cells within the embryo, determining the size of secondary lymphoid organs that are formed ²⁵. In chapter 3 we have demonstrated that intestinal group 3 ILCs, containing the LTi cells, are reduced upon vitamin A deficiency in adult mice. Our data confirmed recently published results, showing reduced numbers of group 3 ILCs in the intestines when vitamin A was absent ^{26,27}. In addition, it is previously reported that the composition of the microbiota is changed in mice that were vitamin A deficient. These changes in the microbiota, especially the reduction in segmented filamentous bacteria, were shown to suppress the generation of Th17 cells ²⁸. Therefore, we should not exclude the role of the changed composition of the microbiota within these vitamin A deficient mice on the ILC population within the lamina propria. However, we were able to demonstrate that group 3 ILCs were directly affected by RA, as mice in which RA signaling was specifically blocked in RORγ⁺ cells, showed reduced numbers of group 3 ILCs similar to vitamin A deficient mice. Moreover, vitamin A deficient mice postnatally developed fewer intestinal lymphoid structures compared to their control counterparts. Our observations illustrate that vitamin A is not only essential for the development of lymphoid tissue during embryogenesis, but also postnatally for the formation of lymphoid clusters within the intestines of adult mice.

Since group 3 ILCs are the main producers of intestinal IL-22, we and others observed diminished expression of IL-22 in the absence of vitamin A as a consequence of the reduced group 3 ILCs ^{26,27}, (chapter 3). These intestinal IL-22 producing ILCs play a crucial role in host defense against *Citrobacter rodentium* infections ²⁹. As a result it has been demonstrated that vitamin A deficient mice were more susceptible to *Citrobacter rodentium* infection, while RA was able to reduce the severity of the infection ^{26,27}. Furthermore, in chapter 4 we demonstrated that BALB/c mice, which are better protected to chemical induced colitis, have a higher vitamin A metabolism compared to C57Bl/6 mice. BALB/c mice showed a higher activity of vitamin A converting enzymes in DCs of the MLN. Consequently the percentages of regulatory T cells present in the intestines and the production of luminal IgA by B cells was increased in BALB/c mice compared to C57Bl/6 mice. Although our data indicate that the difference in vitamin A metabolism between the two different

mouse strains lead to a better protective barrier in BALB/c mice against chemical induced colitis, these mice are also reported to have a different microbiota present in the gut ^{30,31}, which also might influence the disease severity within the mice. Additionally we showed that vitamin A deficient mice were less capable of coping with DSS induced colitis. These data are in line with earlier obtained results, demonstrating a crucial role for vitamin A signaling in maintaining the gut homeostasis ^{26,27,32-34}.

The influence of the microbiota on the mucosal immune system.

Microbiota, which are consistently present in the lumen of the intestines are able to digest the diet and produce metabolites suitable for the host in which they live in symbiosis ^{4,35,36}. While the influence of the microbiota on the immune system of the host is becoming more evident, we demonstrated in chapter 5 that diet derived microbial compounds can modulate the mucosal immune system. Dietary adjustments in the form of fibers showed an increased expression of vitamin A metabolizing enzymes in intestinal epithelial cells, resulting in induced tolerogenic capacity of MLN-DCs. Moreover, we provided evidence that short chain fatty acids (SCFAs), which are fiber derived metabolites, are able to induce RALDH-1 expression in an intestinal epithelial cell line. These SCFAs most likely signal via the inhibition of HDAC, although this should be further addressed with the use of specific HDAC knockout mice.

The role of SCFAs and HDAC inhibition in modulating the mucosal and systemic immune system have been extensively studied in the last decade. SCFAs and HDAC inhibition have been shown to modulate the mucosal immune system with the induction of regulatory T cells, epithelial integrity and additionally may be involved in the secretion of IgA by B-cells ³⁷⁻⁴¹. However, these studies do not address whether there is a role of the epithelial cells in modulating the immune system, even though they are the first to be in contact with environmental factors present in the lumen. In addition, *Allenghat et al.* presented a specific role of HDAC 3 in intestinal epithelial cells for maintaining mucosal homeostasis, and dampening intestinal inflammation ⁴². Together with our data this illustrates a crucial role for environmental factors to influence epithelial cells, which in turn will modulate the mucosal immune system. SCFA mediated signaling can also occur via the binding to G protein coupled receptors (GPCRs). The lack of GPR43 expression in mice resulted in more severe DSS induced colitis with enhanced numbers of neutrophils. In addition, inflammation during arthritis and asthma were also affected ⁴³. These data indicate that SCFAs can affect the mucosal immune system via different pathways, i.e. either via GPCR signaling or through the inhibition of HDAC.

Furthermore, dietary alterations induced differences in the distribution of the microbiota present within the feces of the small intestines. We showed that the presence of specific species of bacteria differed when mice received either a conventional or synthetic diet. Our data, presented in chapter 5, are in line with earlier studies, which compared dietary influences or disease related alterations in the

microbiota. For instance, mice that received either a high fat diet or a low fiber diet showed an enhanced expression of *Erysipelotrichia* bacteria^{44,45}, similar to our mice which were fed a synthetic diet. Patients with inflammatory bowel disease (IBD) often demonstrate alterations in the composition of the microbiota, which correlates with an unhealthy dysbiosis. Therefore, further research on therapeutic innovations, which restores the microbiota distribution in these patients, is very important.

Besides the role of microbiota in the fermentation and production of diet derived products, the mere presence of microbiota can also affect the immune system of the host. Several studies have shown that different cells depend on the presence of bacteria, as germfree or antibiotic treated mice showed alterations in their immune status. For example, the production of IL-1 β by intestinal macrophages, which is critical for Th17 cell development, is dependent on the presence of microbiota^{46,47}. However, the impact of commensal bacteria on ILC development is not completely understood. Although group 1 and group 2 ILCs seem to develop normally in the absence of bacteria^{48,49}, contradictory results have been published concerning the development of ROR γ^+ group 3 ILCs. In the absence of microbiota the development of LTi cells and the formation of secondary lymphoid organs occurs normally within the embryo^{50,51}. Data from adult mice however showed in one study that the presence of microbiota is needed for the production of IL-22 by group 3 ILCs, whereas another study demonstrated that microbiota repressed the production of IL-22 by group 3 ILCs^{52,53}. This indicates that further research is still needed on this topic.

Some species of bacteria have been demonstrated to influence the differentiation of specific cells of the mucosal immune system. Segmented filamentous bacteria were shown to be essential for the induction of Th17 cells. This explained the observation that mice coming from distinct sources have varying numbers of Th17 cells, which correlated with the presence or absence of these bacteria⁵⁴. Moreover, recent studies showed that the specific combinations of several bacteria were able to induce the presence of regulatory T cells, leading to the protection against DSS induced colitis^{55,56}.

It is becoming clear that there is an intricate interaction between microbiota and the mucosal immune system. Therefore, research is now focused on both topics, which led to rapidly increasing insights in the interactions that occur between the microbiota and the host. Nevertheless, additionally research to study the underlying mechanisms that are involved in the generation of mucosal homeostasis is still needed.

Inflammatory bowel disease.

Patients with inflammatory bowel disease (IBD) can be divided into two main groups; patients with Ulcerative Colitis (UC) and patients with Crohn's disease. Both patients suffer from chronic inflammation in the intestinal tract. While UC is mainly located in the colon, Crohn's disease can be found throughout the entire digestive

tract. In both patient groups several studies identified single nucleotide polymorphisms which are associated with an increased risk to develop IBD ^{57,58}. In addition, some correlations have been made between IBD patients and their vitamin A metabolism. For instance, a possible relation is illustrated between a polymorphism in the *Cyp26B1* gene, which codes for an enzyme involved in the breakdown of excessive RA and is a direct target gene of RA signaling, inducing a negative feedback loop. Although, this polymorphism could lead to higher concentrations of RA, this potential link was only seen in Crohn's disease patients and not in UC patients ⁵⁹. Moreover, studies that investigated the capacity to produce RA by both intestinal DC's and macrophages showed an induced enzymatic activity to metabolize vitamin A in Crohn's patients. Nonetheless, macrophage precursors, present in the blood, showed a reduced enzymatic activity to metabolize vitamin A in Crohn's patients compared to healthy controls ⁶⁰. These data indicate that a balanced production of RA is beneficial to induce tolerogenic mechanisms, although excessive production of RA might induce inflammation and can be harmful to the host.

Furthermore, examining the cytokine production of ILCs in Crohn's patients demonstrated an imbalanced ratio of IL-22 and IFN γ producing ILCs, whereas UC patients did not show any differences compared to controls. Crohn's disease was associated with induced IFN γ production, which was mediated via IL-23 production by intestinal macrophages ⁶¹. Paradoxical results were published, demonstrating higher levels of IL-22 in Crohn's patients, while IFN γ levels were not changed compared to healthy controls ⁶². However, while enhanced IL-22 levels were measured in the blood of Crohn's patients, enhanced IFN γ production by ILCs was measured within the intestines. As vitamin A is indicated to influence the differentiation of ILCs and their cytokine production, these data indicate a role for vitamin A in balancing the intestinal ILC subset in Crohn's patients.

In conclusion, although clear results for the protective mechanism induced by vitamin A metabolism or RA in colitis were shown in mouse models ²⁶⁻²⁸, data from patients show that the role of vitamin A metabolism in inflammatory bowel disease is not clear yet and therefore further research is still needed to address these questions.

Colorectal cancer.

Colorectal cancer is one of the most common forms of cancer. Men have a higher risk to develop colorectal cancer compared to females, which increases with age. Other risk factors involved in the development of colorectal cancer are smoking, obesity, family history, physical activity and IBD. Moreover, it is already known for a long time that developing countries have a lower incidence of colorectal cancer compared to western countries ⁶³⁻⁶⁵. Meta-analysis showed that specifically dose dependent fiber intake is inversely associated with the development of colorectal adenoma. Colorectal adenoma risk was reduced by 28% when high fiber intake was analyzed ⁶⁴. The mechanism of dietary fiber intake that has been proposed to medi-

ate protection against colorectal cancer is the effect of fiber derived SCFAs. SCFAs, produced by the microflora, induce hyper activation of the WNT/ β -catenin pathway and consequently promote cell apoptosis in colorectal cancer cells ⁶⁶⁻⁶⁸. Native Africans have higher levels of SCFAs compared to American Africans. Therefore, native Africans from developing countries might have a lower risk to develop colorectal cancer compared to people that live in western countries ⁶⁹. Although clinical trials until now have failed to demonstrate that high-fiber consumption protects against colorectal cancer, a protective effect may have been missed, as these trials were only performed in high-risk populations ⁶⁴. Clinical trials in cancer patients are now focusing on the mechanisms which are induced by dietary fiber ^{70,71}. In addition, high fiber intake can also have beneficial effects in colorectal cancer by influencing the composition of the microbiota and promoting fecal bulking and viscosity, thereby reducing the uptake of carcinogens by intestinal cells ⁷².

In addition, other dietary compounds have been recognized to elevate the risk of colorectal cancer development. For instance, the intake of processed meat was shown to increase the risk of colorectal cancer ⁶⁵. In addition, obesity, which can be caused by a high fat diet, is associated with a higher risk to develop colorectal cancer. Recently it has been demonstrated that a high fat diet, independently of obesity, enhanced the risk for colorectal cancer. The high fat diet altered the composition of the microbiota, which affected the mucosal immune system and showed an impaired response to tumor growth. Moreover, the disease could be transmitted upon microbiota transfer to healthy transgenic mice, which received a normal diet. With the administration of butyrate the microbiota composition was restored and tumor growth was reduced ⁷³. Thus, the fermentation of dietary products executed by the microbiota is not only important for controlling colorectal cancer, it also affects the composition of the microbiota.

As we have demonstrated that changes in diet and microbiota composition affected the vitamin A metabolism in the intestine (chapter 5) and in light of the important association of microbiota and colorectal cancer, vitamin A metabolism might play a crucial role in the development of colorectal cancer. Material of patients with colorectal cancer demonstrated a higher expression of Cyp26a1, Cyp26B1 and LRAT compared to healthy controls. These enzymes are all involved in the degradation of either retinol or RA. A high expression of Cyp26B1 and LRAT were even associated with poor prognosis in colorectal patients ⁷⁴. Although they concluded within this article that induced levels of vitamin A or RA degrading enzymes would lead to lower levels of RA, these data also might illustrate that patients have higher levels of RA as these enzymes are induced by RA signaling itself. Nevertheless, an association for vitamin A metabolism and colorectal cancer is indicated.

Environmental factors in other diseases.

Type 2 diabetes is a metabolic disorder that is caused by insulin resistance of the body, leading to high sugar levels in blood. This disorder is associated with both ge-

netic and environmental factors. Recently, with the use of metagenome wide association studies, two research groups were able to identify specific microbial changes in type 2 diabetic patients. Although some differences were found, which could be related to the distinct background of the patients studied, being either from European or Chinese origin, both studies showed specific reduced presence of butyrate producing bacteria. These observations demonstrated a predictive potential of identifying type 2 diabetic patients by the intestinal microbiome^{75,76}. Moreover, one study was able to discriminate between pre-type 2 diabetes and normal glucose tolerance with metagenomic wide association research⁷⁵. The data illustrated a critical role for the microbiota in the development of type 2 diabetes and suggest that patients can be treated by restoring their dysbiotic microbiota.

Other metabolic disorders, which are associated with an altered intestinal microbiota, are obesity and cardiovascular diseases⁵. When the mucosal barrier is defective an endotoxaemia may be induced which will further promote the development of metabolic disorders. Therefore, the production of IL-22 by intestinal ILCs and T cells is essential to maintain the mucosal barrier, as it modulates epithelial barrier functions. It has been demonstrated that mice which have a defect in IL-22 signaling, have indeed higher chances to develop metabolic disorders when they receive a high fat diet. In addition, treatment with IL-22 has been shown to overcome insulin resistance⁷⁷, illustrating that therapeutic intervention aimed at regulating the IL-22 pathway might be beneficial for patients with metabolic disorders. Vitamin A metabolites might thus be very interesting as they enhance IL-22 production^{26,27}, (chapter 3).

Intake of dietary fibers has not only been shown to benefit intestinal disorders, as also many other diseases were shown to be dampened by it. Systemic higher levels of SCFAs, which are produced by the microbiota from dietary fibers, showed reduced inflammation in allergic lung diseases. When these findings were further analyzed an enhanced hematopoiesis of DC precursors in the bone marrow was demonstrated, with less activation in the lung upon allergic antigen encounter⁴⁵. These results showed a significant role for systemic levels of SCFAs induced by fiber intake in allergic lung diseases.

Furthermore, obesity, diabetes and cardiovascular diseases are shown to have beneficial effects from a high fiber intake. First of all, dietary fibers are related to a better body mass index by reducing weight. Secondly, fibers regulate the microbiota composition, which often is disbalanced in these patients. And moreover, dietary fiber intake is associated with anti-inflammatory effects⁷⁸. In addition, it has even demonstrated that dietary fiber intake and microbiota composition influences behavior and cognition in both human and mice^{78,79}.

In sum, not only intestinal disorders are influenced by dietary intake and microbiota composition, but also many other conditions and diseases.

Therapeutic implications.

Treatment of microbiota dysbiosis in patients with intestinal disorders reduces clinical symptoms. Additionally, microbiota treatment might even help the systemic immune system in other diseases as type 2 diabetes. While antibiotics were first associated with benefits in IBD patients, it has been demonstrated that antibiotic exposure further enhances the microbiota dysbiosis in IBD patients. Moreover, antibiotic resistance is a growing health concern and therefore antibiotic treatment in a chronic disease is not preferred⁸⁰. With the increased interest in the field of microbiota and their role in regulating the mucosal immune system new insights are revealed. Data are now emerging which specific bacteria or their products might have beneficial effects for the host. Therefore, prebiotics and probiotics are giving new possible therapeutic approaches. While prebiotics stimulate the growth of specific bacteria, probiotics are live bacteria, which both, after intake, should induce health benefits to the host. Not only the live bacteria might have beneficial effects, also bacterial components, dead bacteria and the substances secreted by bacteria have demonstrated health benefits for the host^{81,82}. However, clinical trials showed moderate effects of probiotic treatments, which might be because these treatments are not standardized yet. The optimal dosing and efficacy of certain prebiotics and probiotics should be further investigated in large scale clinical trials^{83,84}. Furthermore, single strain probiotics might have limited effects, while combinations of a few bacteria have a better potential to sustain within the microflora of the host. Several studies demonstrated promising results with combinations of a few bacteria, which were able to induce regulatory T cells and protection against DSS induced colitis in mice with a dysbiotic microbiota^{55,56}. A more resolute treatment would be fecal transplantation. After the right donor is selected, which most of the time will be within the direct surroundings of the patient, the fecal sample will be screened for unknown pathogens. Although this method has shown good results in infection with *Clostridium difficile*, fecal transplantation is an intense treatment for the patient as first the total intestines will be flushed followed by fecal transplantation^{56,85}. Besides therapeutics that directly modulate the microbiota distribution, dietary intake also effects this distribution. Moreover, the diet can both directly and indirectly, after microbiota fermentation, affect the mucosal immune system. Whereas it might be one of the oldest concepts in medicine to promote health via the diet, only within the last decade we now start to understand partly how these mechanisms work. Intake of high fibers are associated with a reduced risk to develop intestinal disorders, but has also been shown to have protective effects against many other diseases. While the recommended fiber intake is 25-28 gram a day, the average American only consumes around 16 gram of fibers a day⁸⁶. This already indicates that the average fiber intake is often too low. Additionally, nutrition in the form of vitamins and Ahr ligands are shown to be beneficial for the host by modulating

the mucosal immune system. Therefore, a healthy varied diet with a high fiber and vitamin intake would stimulate a symbiotic microbiota and modulate the mucosal immune system to be tolerant against harmless antigens^{78,87}.

Concluding remarks.

Overall, within this thesis we have described several processes which modulate the mucosal immune system. We have demonstrated additional affects, which are regulated by vitamin A metabolism, that are of influence for the functioning of the mucosal immune system. Moreover, we presented data that showed both genetic factors and dietary fibers were able to regulate the vitamin A metabolism in the intestines. These results provide new therapeutic strategies to restore and maintain a balanced mucosal immune system by adapting dietary intake and modulate the microbiota. This might not only be helpful for patients with intestinal disorders but also various other inflammatory and metabolic diseases.

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